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(54) Title: METHODS AND COMPOSITIONS FOR PRODUCTION OF PLANT FOODSTUFFS WITH ENHANCED SWEET COMPONENT OF FLAVOR

(57) Abstract

The invention discloses a transgenic method for producing plant foodstuffs such as fruits, vegetables or seeds with a modified sweet component. It involves the temporal expression of a gene which encodes the sweet protein brazzein to enhance the sweet component of flavor. The invention also includes constructs, vectors, and transgenic plants for production of such foodstuffs.

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TITLE: METHODS AND COMPOSITIONS FOR PRODUCTION OF PLANT FOODSTUFFS WITH ENHANCED SWEET COMPONENT OF FLAVOR

## FIELD OF THE INVENTION

This invention relates generally to the field of plant molecular biology and in particular to transgenic plants, seeds, and tissues which have been genetically modified to create plants that produce edible foodstuffs with an enhanced sweet flavor component.

## BACKGROUND OF THE INVENTION

Fresh fruits and vegetables are popular food items with consumers. During winter months, however, the majority of areas around the country are unable to grow their own fruits and vegetables and must therefore rely on importation of these items from the southern portions of the United States. The fresh fruit and vegetable industries are especially important to these southern states, including Florida, Georgia, Texas, and California, which rely on these industries as a primary source of income.

Generally, the two most desirable components of fresh fruits and vegetables are flavor, which is usually the level of sweetness, and appearance. Sweetness is determined by the amount of soluble solids (sweetness component) and the degree of acidity in the fruit or vegetable. Several investigators have reported that flavor differences correlate with soluble solids and with the ratio of soluble solids to titratable acid. Soluble solids are measured as °Brix with a bench top model ABBE -3L Baush & Lombe refractometer according to methods of Stevens et al, "Genotypic Variation for Flavor and Composition in Fresh Market Tomatoes", J. Amer. Soc. Hort. Sci. (1977) 105(5) 680-689. Other measurements of sweetness include acidity and reducing sugar which are also disclosed. The most important soluble solids consist of the sugars sucrose, fructose, and glucose. The amount of sweetness is dependent upon environmental factors such as lack of sun and cool temperatures, both of which decrease the amount of sweetness component produced by the fruit or vegetable.

In general, fruit will have a °Brix of 8 to 9 in the summer months and 5 to 6 in the winter. This °Brix can fall even lower if the winter is unseasonably cool or rainy since this further decreases the production of sweetness component. For many fruits and vegetables, once the sweetness level falls

below 8, the degree of sweetness is often unsatisfactory to the taste and they are therefore unacceptable for consumer consumption.

Furthermore, some fruits require a °Brix of as high as 14 for optimal taste. These fruits include the various types of melons, such as watermelon, cantaloupe, and honeydew. Typical °Brix measurements in these fruits are 12 in the summer months and 8 in the winter. If the °Brix falls below 8, these fruits take on a cardboard-like taste. Thus, during inclement weather, it is even more difficult for these fruit growers to keep the °Brix at a sufficient level so that the fruit maintains an acceptable taste.

There is therefore a need in the art for a method of increasing the sweetness of fruits and vegetables during the winter months and periods of inclement weather conditions wherein the sweetness of these fruits and vegetables falls to an unacceptable level, or to enhance the flavor of foodstuffs generated from plants even in the optimum growing conditions.

Current methods for increasing the sweetness of fruits and vegetables include supplementing the sweetness component with an exogenous sweetener. There were several problems associated with this approach, however. The most widely used natural sweetener, sugar, has several problems associated with its use, including high caloric content, association with tooth decay, and undesirability for diabetic use.

Artificial sweeteners offer an alternative but are also deficient in certain respects. For example, aspartame (Nutrasweet®) loses its sweetness when exposed to elevated temperatures for long periods. This makes aspartame unsuitable for use in most cooking or baking applications of the fruits and vegetables. Another problem associated with artificial sweeteners is their temporal sweetness profiles in respect to sugar. In general, the level of sweetness in artificial sweeteners usually fades sooner in comparison to sugar.

Previously, the natural sweet component in foods was attributed only to carbohydrates, such as sucrose, fructose, and glucose. It was not contemplated that macromolecules such as proteins could elicit taste activities similar to small ones, i.e. sugars.

Researchers then discovered the existence of miraculin, a sweet tasting protein which acts by adding sweetness to a sour taste. Kurihara, et al. (1968) Science 161, 1241-1243. This discovery was followed by the isolation of two sweet tasting proteins: monellin and thaumatin, which in themselves taste sweet. van der Wel, H., et al., (1991) Development in Food Proteins, vol. 4, pp. 219-245. Monellin is an intensely sweet polypeptide isolated from the West African plant *Dioscoreophyllum comminisil.* Thaumatin is an isolate from

*Thaumatococcus daniellii*, a West African plant having triangular shaped fruit at ground level and has an average sweetness 2500 times that of sucrose.

More recently, other sweet proteins have been isolated. These sweet proteins include curculin (Harada, S., et al., (1994) J. Mol. Biol. 238, 286-287), mabinlin (Ming, D., et al. (1986) Acta Botanica Yunnanica 8, 2, 181-192), pentadin (van der Wel, H., et al., (1989) Chem. Senses 14, 75-78), and brazzein (Ming, D., et al., FEBS Letters 355 106-108), the latter being isolated from *Pentadiplandra brazzeana* Baillon. Brazzein is smaller than thaumatin and is also much sweeter at higher concentrations.

Several of these sweet proteins have been purified and characterized. For example, U.S. Patent No. 3,998,798 to Cagan et al. discloses a purified form of the sweet protein monellin. Frank, et al., (1976) Hoppe-Seyler's Z. Physiol. Chem. 357, 585-592 disclose the amino acid sequence of both chains of monellin. U.S. Patent No. 4,891,316 to Verrips et al. sets forth the DNA sequences encoding the various forms of mature thaumatin and well as cloning vehicles containing the DNA and their use in transforming microorganisms. Further, Ming et al., FEBS Letters 355, 106-108 and U.S. Patent Nos. 5,326,580 and 5,346,998 have characterized the amino acid sequence and DNA sequence of brazzein. The disclosures of the above references are herein specifically incorporated by reference. Use of these sweet proteins has focused primarily on their addition to already harvested plant foodstuffs.

It is therefore a primary objective of the present invention to endogenously supplement the sweet component of flavor of fruits and vegetables by use of expression constructs encoding sweet proteins.

It is another objective of the present invention to modify the sweet component of fruits and vegetables such that 1% of the fruits and vegetables overall 1% protein content is sweet protein.

It is a further objective of the present invention to modify the sweet component of fruits and vegetables by linking the DNA sequence encoding a sweet protein to a promoter in order to express the protein sequence in a transgenic plant.

It is yet another objective of the present invention to provide inbred parental lines which can be crossbred resulting in an $F_1$ hybrid plant which will produce fruits and vegetables containing a modified sweet component.

It is another objective of the present invention to provide vehicles for transformation of plant cells including viral or plasmid vectors incorporating the genes and promoters of the invention.

It is another objective of the present invention to provide bacterial cells comprising such vectors for maintenance of replication and plant transformation.

These and other objectives will become obvious from the foregoing description of the invention which follows.

## SUMMARY OF THE INVENTION

The invention provides plant derived foodstuffs which are endogenously sweetened and improved in flavor by production of brazzein in the component of the plant from which the foodstuffs are derived. Accordingly, in one aspect, the invention is directed to expression systems capable of production of brazzein. According to the invention, a gene encoding brazzein is operably linked to a regulatory promoter which can direct expression of the sweet protein gene developmentally and spatially to the edible portion of the plant thus forming an expression construct which can be used to transform plant cells.

For example, fruits with enhanced sweet taste can be produced by placing brazzein under control of promoters which are activated during the ripening process. Seeds can be sweetened by using promoters for seed storage proteins as promoters for the brazzein expression construct. Vegetable portions of plants can be sweetened by introduction of brazzein under control of sequences which operate as constitutive promoters or by using organ specific promoters targeted to the edible organ of interest (root, stem, leave etc.) Inducible promoters can also be used to provide for control of expression upon application of inducer agents. Thus the transformed plants can be modified in controlled ways to provide enhanced sweetness at the desired locations and the desired state of development.

In other aspects, the invention is directed to plant cells transformed with the expression systems described above, to plants regenerated from or containing these cells, to edible portions of these plants, and to foodstuffs prepared from them. In other aspects, the invention is directed to methods to produce fruits, seeds and vegetables with enhanced sweetness which method comprises cultivation of the transgenic plants of the invention followed by recovery of the desired edible portions.

## DETAILED DESCRIPTION OF THE INVENTION

According to the invention herein, rather than providing the sweetener proteins as independent products, the coding sequences for brazzein can be

inserted into specialized expression control DNA sequences which are compatible with higher plants used to obtain transgenic plants which create naturally sweetened plant products. Although the primary effect is that of sweetening, it is understood that this can affect the overall flavor and cause general improvement in taste. In these embodiments, control regions which are functional either constitutively inducibly or in specialized tissues in plants are employed.

In the description of the invention that follows, a number of terms are used extensively. The following definitions are provided in order to remove ambiguities in the intent or scope of their usage in the specification and claims, and to facilitate understanding of the invention.

As used herein, the term "fruit" shall include any angiosperm plant which has its pollen and ovule producing organs in flowers; with ovules enclosed in an ovary, and after fertilization with each ovule developing into a seed while the ovary expands into a fruit. The term "fruit" is also intended to include tomatoes, peppers and other such fruits which are commonly referred to as vegetables.

As used herein, the term "vegetable" includes herbaceous plants having an edible part or parts, including the leaves, stems, seeds and seed pods, flowers, roots and tubers.

As used herein, the term "brazzein" refers to a sweet protein disclosed in United States patents 5,326,580 and 5,346,998 as well as sweet proteins which are substantially equivalent thereto.

"Substantially equivalent" as used herein means that the peptide is a substance having a sweetening power at least 50 times that of sucrose and at least 30%-50% homology with at least one form of the native brazzein protein as disclosed in United States Patents 5,326,580 and 5,346,998 previously incorporated by reference. 80% homology is preferred and 90% homology is most preferred especially including conservative substitutions.

Homology is calculated by standard methods which involve aligning two sequences to be compared so that maximum matching occurs, and calculating the percentage of matches. Substantially equivalent substances to these include those wherein one or more of the residues of the native sequence is deleted, substituted for, or inserted by a different amino acid or acids.

Preferred substitutions are those which are conservative, i.e., wherein a residue is replaced by another of the same general type. As is well understood, naturally occurring amino acids can be sub classified as acidic, basic, neutral and polar, or neutral and nonpolar. Furthermore, three of the encoded amino

acids are aromatic. It is generally preferred that peptides differing from the native brazzein sequence contain substitutions which are from the same group as that of the amino acid replaced. Thus, in general, the basic amino acids Lys and Arg are interchangeable; the acidic amino acids aspartic and glutamic are interchangeable; the neutral polar amino acids Ser, Thr, Cys, Gln, and Asn are interchangeable; the nonpolar aliphatic acids Gly, Ala, Val, Ile, and Leu are conservative with respect to each other (but because of size, Gly and Ala are more closely related and Val, Ile and Leu are more closely related), and the aromatic amino acids Phe, Trp, and Tyr are interchangeable. While proline is a nonpolar neutral amino acid, it represents difficulties because of its effects on conformation, and substitutions by or for proline are not preferred, except when the same or similar conformational results can be obtained. Polar amino acids which represent conservative changes include Ser, Thr, Gln, Asn; and to a lesser extent, Met. In addition, although classified in different categories, Ala, Gly, and Ser seem to be interchangeable, and Cys additionally fits into this group, or may be classified with the polar neutral amino acids. Some substitutions by amino acids from different classes may also be useful to modify sweet taste responses.

In general, whatever substitutions are made are such that the sweetness of the intact proteinaceous molecule is retained and ancillary properties, such as non-toxicity are not substantially disturbed.

It should be further noted that if the protein embodiments of the invention are produced recombinantly as intracellular proteins, an N-terminal methionine residue may be retained in the finished product. Cleavage of this N-terminal methionine to liberate the native sequence may or may not be complete. In addition, the sweetening peptide or protein may be produced as a fusion protein with additional heterologous upstream or downstream sequence.

A "structural gene" is a DNA sequence that is transcribed into messenger RNA (mRNA) which is then translated into a sequence of amino acids characteristic of a specific polypeptide.

A "promoter" is a DNA sequence that directs the transcription of a structural gene. Typically, a promoter is located in the 5' region of a gene, proximal to the transcriptional start site of a structural gene.

The term "expression" refers to biosynthesis of a gene product. Structural gene expression involves transcription of the structural gene into mRNA and then translation of the mRNA into one or more polypeptides.

A "cloning vector" is a DNA molecule such as a plasmid, cosmid, or bacterial phage that has the capability of replicating autonomously in a host cell. Cloning vectors typically contain one or a small number of restriction endonuclease recognition sites at which foreign DNA sequences can be inserted in a determinable fashion without loss of essential biological function of the vector, as well as a marker gene that is suitable for use in the identification and selection of cells transformed with the cloning vector. Marker genes typically include genes that provide tetracycline resistance or ampicillin resistance.

An "expression vector" is a DNA molecule comprising a gene that is expressed in a host cell. Typically, gene expression is placed under the control of certain regulatory elements including promoters, tissue specific regulatory elements, and enhancers. Such a gene is said to be "operably linked to" the regulatory elements.

A "recombinant host" may be any prokaryotic or eukaryotic cell that contains either a cloning vector or an expression vector. This term also includes those prokaryotic or eukaryotic cells that have been genetically engineered to contain the clone genes in the chromosome or genome of the host cell.

A "transgenic plant" is a plant having one or more plant cells that contain an expression vector. Plant tissue includes differentiated and undifferentiated tissues or plants, including but not limited to roots, stems, shoots, leaves, pollen, seeds, tumor tissue, and various forms of cells and culture such as single cells, protoplasm, embryos, and callus tissue. The plant tissue may be in plant or in organ, tissue, or cell culture.

The invention in one aspect comprises expression constructs comprising a DNA sequence which encodes upon expression a brazzein sweet protein operably linked to a promoter to direct expression of the protein. These constructs are then introduced into plant cells using standard molecular biology techniques. The invention can be also be used for hybrid plant or seed production, once transgenic inbred parental lines have been established.

Sweet proteins are very potent with a sweetness taste nearly 1000 times that of sucrose. Brazzein is particularly useful as a sweet protein because of its beneficial chemical and biological properties. Brazzein is even sweeter than most known sweet proteins, including thaumatin. Its taste differs from that of thaumatin, it has more phasic response and a faster adapting tonic phase. The molecular weight of brazzein is 6473 Da, which is less than thaumatin ($M_r$ 22,206) monellin ($M_r$ 11086), curculin ($M_r$ 12,491), and

mabinlin ($M_r$ 12,441). Thus brazzein is the smallest protein sweetener known. Brazzein is also extremely thermostable: all sweet proteins except mabinlin lose their sweetness in a few minutes at 80°C but brazzein's sweetness remains for hours at that temperature.

The methods of the invention described herein may be applicable to any species of plant, the fruit or other organ of which is edible or within which it is desirable to make the flavor sweeter. Fruits and vegetable plants which can be made sweeter according to the methods of the invention include but are not limited to include, melons such as cantaloupe, honeydew and watermelon, and musk melon, berries such as strawberries, and blueberries, peppers such as green peppers, red bell peppers, yellow peppers, carrots, tomatoes, oranges, plums, alfalfa, squash, eggplant, sweetcorn, peas, onions, lettuce, avocados, mangos, papayas, nectarines, apples, grapefruit, lemons, limes, tangerines, pears and peaches.

Preferred fruits and vegetables include melons, tomatoes, carrots, onions, and sweetcorn. For example, the invention is especially useful for onions wherein the sweetness component is important but often contributes to its poor storage capabilities. By selectively substituting sweet protein for its normal carbohydrate sweetness component, the vegetable will be less susceptible to degradation and storage time will improve.

The most preferred fruits for use in the invention are melons. While the sweetness of most fruits and vegetables can be attributed to its sweetness component and degree of acidity, melons lack the acidity component and, therefore, rely on their sweetness components exclusively for flavor. Because of this, its °Brix should be at least 12, with 14 being preferred for ultimate flavor. During the winter months, the °Brix of melons often drops to 8 or below, giving the fruit a cardboard-like taste. By supplementing the sweet component, melons can be more successfully grown in the winter with better flavor.

Production of a genetically modified plant tissue expressing a structural gene under the control of regulatory promoters combines teachings of the present disclosure with a variety of techniques and expedients known in the art. In most instances, alternate expedients exist for each stage of the overall process. The choice of expedients depends on the variables such as the plasmid vector system chosen for the cloning and introduction of the recombinant DNA molecule, the plant species to be modified, the particular structural gene, promoter elements and upstream elements used. Persons skilled in the art are able to select and use appropriate alternatives to achieve

functionality. Culture conditions for expressing desired structural genes and cultured cells are known in the art. Also as known in the art, a number of both monocotyledonous and dicotyledonous plant species are transformable and regenerable such that whole plants containing and expressing desired genes under regulatory control of the promoter molecules according to the invention may be obtained. As is known to those of skill in the art, expression in transformed plants may be tissue specific and/or specific to certain developmental stages. Truncated promoter selection and structural sweet protein gene selection are other parameters which may be optimized to achieve desired plant expression as is known to those of skill in the art and taught herein.

The promoters or control systems used in the methods of the invention may include a fruit or organ specific promoter. an inducible promoter or a constitutive promoter.

A large number of suitable promoter systems are available. For example one constitutive promoter useful for the invention is the cauliflower mosaic virus (CaMV) 35S. It has been shown to be highly active in many plant organs and during many stages of development when integrated into the genome of transgenic plants including tobacco and petunia, and has been shown to confer expression in protoplasts of both dicots and monocots.

Organ-specific promoters are also well known. For example, the E8 promoter is only transcriptionally activated during tomato fruit ripening, and can be used to target gene expression in ripening tomato fruit (Deikman and Fischer, EMBO J. (1988) 7:3315; Giovannoni et al., The Plant Cell (1989) 1:53). The activity of the E8 promoter is not limited to tomato fruit, but is thought to be compatible with any system wherein ethylene activates biological processes. Similarly the Lipoxegenase ("the LOX gene") is a fruit specific promoter.

Other fruit specific promoters are the 1.45 promoter fragment disclosed in Bird, et,al Plant Mol. Bio, pp 651-663(1988) and the polygalacturonase promoter from tomato disclosed in U.S. Patent 5,413,937 to Bridges et al.
Leaf specific promoters include as the AS-1 promoter disclosed in US Patent 5,256,558 to Coruzzi and the RBCS-3A promoter isolated from pea the RBCS-3A gene disclosed in US Patent 5,023,179 to Lam et al.

And finally root specific promoters include the Cam 35 S promoter disclosed in US Patent 391,725 to Coruzzi et al; the RB7 promoter disclosed in US patent 5,459,252 to Conking et al and the promoter isolated from Brassica

Napus disclosed in US Patent 5,401, 836 to Bazczynski et al. which give root specific expression.

Other examples of promoters include maternal tissue promoters such as seed coat, pericarp and ovule. Promoters highly expressed early in endosperm development are most effective in this application. Of particular interest is the promoter from the a' subunit of the soybean β-conglycinin gene [Walling et al., Proc. Natl. Acad. Sci. USA 83:2123-2127 (1986)] which is expressed early in seed development in the endosperm and the embryo.

Further seed specific promoters include the Napin promoter described in united States Patent 5,110,728 to Calgene, which describes and discloses the use of the napin promoter in directing the expression to seed tissue of an acyl carrier protein to enhance seed oil production; the DC3 promoter from carrots which is early to mid embryo specific and is disclosed at Plant Physiology, Oct. 1992 100(2) p. 576-581, "Hormonal and Environmental Regulation of the Carrot Lea-class Gene Dc 3, and Plant Mol. Biol., April 1992, 18(6) p. 1049-1063, "Transcriptional Regulation of a Seed Specific Carrot Gene, DC 8": the phaseolin promoter described in United States Patent 5,504,200 to Mycogen which is hereby which discloses the gene sequence and regulatory regions for phaseolin, a protein isolated from *P. vulgaris* which is expressed only while the seed is developing within the pod, and only in tissues involved in seed generation.

Other organ-specific promoters appropriate for a desired target organ can be isolated using known procedures. These control sequences are generally associated with genes uniquely expressed in the desired organ. In a typical higher plant, each organ has thousands of mRNAs that are absent from other organ systems (reviewed in Goldberg, Phil, Trans. R. Soc. London (1986) B314-343. mRNAs are first isolated to obtain suitable probes for retrieval of the appropriate genomic sequence which retains the presence of the natively associated control sequences. An example of the use of techniques to obtain the cDNA associated with mRNA specific to avocado fruit is found in Christoffersen et al., Plant Molecular Biology (1984) 3:385. Briefly, mRNA was isolated from ripening avocado fruit and used to make a cDNA library. Clones in the library were identified that hybridized with labeled RNA isolated from ripening avocado fruit, but that did not hybridize with labeled RNAs isolated from unripe avocado fruit. Many of these clones represent mRNAs encoded by genes that are transcriptionally activated at the onset of avocado fruit ripening.

The promoter used in the method of the invention may be an inducible promoter. An inducible promoter is a promoter that is capable of directly or indirectly activating transcription of a DNA sequence in response to an inducer. In the absence of an inducer, the DNA sequence will not be transcribed. Typically, the protein factor that binds specifically to an inducible promoter to activate transcription is present in an inactive form which is then directly or indirectly converted to the active form by the inducer. The inducer may be a chemical agent such as a protein, metabolite (sugar, alcohol etc.), a growth regulator, herbicide, or a phenolic compound or a physiological stress imposed directly by heat, salt, toxic elements etc. or indirectly through the action of a pathogen or disease agent such as a virus. A plant cell containing an inducible promoter may be exposed to an inducer by externally applying the inducer to the cell such as by spraying, watering, heating, or similar methods. Examples of inducible promoters include the inducible 70 kd heat shock promoter of D. melanogaster (Freeling, M., Bennet, D.C., Maize ADN 1, Ann. Rev. of Genetics 19:297-323) and the alcohol dehydrogenase promoter which is induced by ethanol (Nagao, R.T., et al., Miflin, B.J., Ed. Oxford Surveys of Plant Molecular and Cell Biology, Vol. 3, p. 384-438, Oxford University Press, Oxford 1986) or the Lex A promoter which is triggered with chemical treatment and is available through Ligand pharmaceuticals. The inducible promoter may be in an induced state throughout seed formation or at least for a period which corresponds to the transcription of the DNA sequence of the recombinant DNA molecule(s).

Another example of an inducible promoter is the chemically inducible gene promoter sequence isolated from a 27 kd subunit of the maize glutathione-S-transferase (GST II) gene. Two of the inducers for this promoter are N,N-diallyl-2,2-dichloroacetamide (common name: dichloramid) or benzyl-=2-chloro-4-(trifluoromethyl)-5-thiazolecarboxylate (common name: flurazole). In addition, a number of other potential inducers may be used with this promoter as described in published PCT Application No. PCT/GB90/00110 by ICI.

Another example of an inducible promoter is the light inducible chlorophyll a/b binding protein (CAB) promoter, also described in published PCT Application No. PCT/GB90/00110 by ICI.

Inducible promoters have also been described in published Application No. EP89/103888.7 by Ciba-Geigy. In this application, a number of inducible promoters are identified, including the PR protein genes, especially the tobacco PR protein genes, such as PR-1a, PR-1b, PR-1c, PR-1, PR-A, PR-S, the

cucumber chitinase gene, and the acidic and basic tobacco beta-1,3-glucanase genes. There are numerous potential inducers for these promoters, as described in Application No. EP89/103888.7.

The preferred promoters may be used in conjunction with naturally occurring flanking coding or transcribed sequences of the sweet protein encoding genes or with any other coding or transcribed sequence that is critical to sweet protein formation and/or function.

It may also be desirable to include some intron sequences in the promoter constructs since the inclusion of intron sequences in the coding region may result in enhanced expression and specificity. Thus, it may be advantageous to join the DNA sequences to be expressed to a promoter sequence that contains the first intron and exon sequences of a polypeptide which is unique to cells/tissues of a plant critical to sweet protein formation and/or function.

Additionally, regions of one promoter may be joined to regions from a different promoter in order to obtain the desired promoter activity resulting in a chimeric promoter. Synthetic promoters which regulate gene expression may also be used. The expression system may be further optimized by employing supplemental elements such as transcription terminators and/or enhancer elements.

In addition to a promoter sequence, the expression cassette should also contain a transcription termination region downstream of the structural gene to provide for efficient termination. The termination region or polyadenylation signal may be obtained from the same gene as the promoter sequence or may be obtained from different genes. Polyadenylation sequences include, but are not limited to the *Agrobacterium* octopine synthase signal (Gielen et al., EMBO J. (1984) 3:835-846) or the nopaline synthase signal (Depicker et al., Mol. and Appl. Genet. (1982) 1:561-573).

Recombinant DNA molecules containing any of the DNA sequences and promoters described herein may additionally contain selection marker genes which encode a selection gene product which confer on a plant cell resistance to a chemical agent or physiological stress, or confers a distinguishable phenotypic characteristic to the cells such that plant cells transformed with the recombinant DNA molecule may be easily selected using a selective agent. One such selection marker gene is neomycin phosphotransferase (NPT II) which confers resistance to kanamycin and the antibiotic G-418. Cells transformed with this selection marker gene may be selected for by assaying for the presence in vitro of phosphorylation of kanamycin using techniques

described in the literature or by testing for the presence of the mRNA coding for the NPT II gene by Northern blot analysis in RNA from the tissue of the transformed plant. Other commonly used selection markers include the ampicillin resistance gene, the tetracycline resistance and the hygromycin resistance gene. Transformed plant cells thus selected can be induced to differentiate into plant structures which will eventually yield whole plants. It is to be understood that a selection marker gene may also be native to a plant.

A recombinant DNA molecule containing any of the DNA sequences and promoters described herein may be integrated into the genome of a plant by first introducing a recombinant DNA molecule into a plant cell by any one of a variety of known methods. Preferably the recombinant DNA molecule(s) are inserted into a suitable vector and the vector is used to introduce the recombinant DNA molecule into a plant cell.

The use of Cauliflower Mosaic Virus (CaMV) (Howell, S.H., et al, 1980, Science 208:1265) and gemini viruses (Goodman, R.M., 1981, J. Gen Virol. 54:9) as vectors has been suggested but by far the greatest reported successes have been with Agrobacteria sp. (Horsch, R.B., et al, 1985, Science 227:1229-1231).

Methods for the use of Agrobacterium based transformation systems have now been described for many different species. Generally strains of bacteria are used that harbor modified versions of the naturally occurring Ti plasmid such that DNA is transferred to the host plant without the subsequent formation of tumors. These methods involve the insertion within the borders of the Ti plasmid the DNA to be inserted into the plant genome linked to a selection marker gene to facilitate selection of transformed cells. Bacteria and plant tissues are cultured together to allow transfer of foreign DNA into plant cells then transformed plants are regenerated on selection media. Any number of different organs and tissues can serve as targets from Agrobacterium mediated transformation as described specifically for members of the Brassicaceae. These include thin cell layers (Charest, P.J., et al, 1988, Theor. Appl. Genet. 75:438-444), hypocotyls (DeBlock, M., et al, 1989, Plant Physiol. 91:694-701), leaf discs (Feldman, K.A., and Marks, M.D., 1986, Plant Sci. 47:63-69), stems (Fry J., et al, 1987, Plant Cell Repts. 6:321-325), cotyledons (Moloney M. M., et al, 1989, Plant Cell Repts. 8:238-242) and embryoids (Neuhaus, G., et al, 1987, Theor. Appl. Genet. 75:30-36). It is understood, however, that it may be desirable in some crops to choose a different tissue or method of transformation.

Other methods that have been employed for introducing recombinant molecules into plant cells involve mechanical means such as direct DNA uptake, liposomes, electroporation (Guerche, P. et al, 1987, Plant Science 52:111-116) and micro-injection (Neuhaus, G., et al, 1987, Theor. Appl. Genet. 75:30-36). The possibility of using microprojectiles and a gun or other device to force small metal particles coated with DNA into cells has also received considerable attention (Klein, T.M. et al., 1987, Nature 327:70-73).

It is often desirable to have the DNA sequence in homozygous state which may require more than one transformation event to create a parental line, requiring transformation with a first and second recombinant DNA molecule both of which encode the same gene product. It is further contemplated in some of the embodiments of the process of the invention that a plant cell be transformed with a recombinant DNA molecule containing at least two DNA sequences or be transformed with more than one recombinant DNA molecule. The DNA sequences or recombinant DNA molecules in such embodiments may be physically linked, by being in the same vector, or physically separate on different vectors. A cell may be simultaneously transformed with more than one vector provided that each vector has a unique selection marker gene. Alternatively, a cell may be transformed with more than one vector sequentially allowing an intermediate regeneration step after transformation with the first vector. Further, it may be possible to perform a sexual cross between individual plants or plant lines containing different DNA sequences or recombinant DNA molecules preferably the DNA sequences or the recombinant molecules are linked or located on the same chromosome, and then selecting from the progeny of the cross, plants containing both DNA sequences or recombinant DNA molecules.

Expression of recombinant DNA molecules containing the DNA sequences and promoters described herein in transformed plant cells may be monitored using Northern blot techniques and/or Southern blot techniques known to those of skill in the art.

The expression of the DNA should be controlled so that the sweet protein is expressed in the fruit or vegetable portion of the plant, and not in inedible portions, such as the leaves. Such gene expression techniques are well known to those skilled in the art of recombination techniques. A large number of plants have been shown capable of regeneration from transformed individual cells to obtain transgenic whole plants. For example, regeneration has been shown for dicots as follows:

apple, Malus pumila (James et al., Plant Cell Reports (1989) 7:658);

blackberry, *Rubus*, Blackberry/raspberry hybrid, *Rubus*, red raspberry, *Rubus* (Graham et al., Plant Cell, Tissue and Organ Culture (1990) 20:35);

carrot, *Daucus carota* (Thomas et al., Plant Cell Reports (1989) 8:354; Wurtele and Bulka, Plant Science (1989) 61:253);

cauliflower, *Brassica oleracea* (Srivastava et al., Plant Cell Reports (1988) 7:504);

celery, *Apium graveolens* (Catlin et al., Plant Cell Reports (1988) 7:100);

cucumber, *Cucumis sativus* (Trulson et al., Theor. Appl. Genet. (1986) 73:11);

eggplant, *Solanum melonoena* (Guri and Sink, J. Plant Physiol. (1988) 133:52)

lettuce, *Lactuca sativa* (Michelmore et al., Plant Cell Reports (1987) 6:439);

potato, *Solanum tuberosum* (Sheerman and Bevan, Plant Cell Reports (1988) 7:13);

rape, *Brassica napus* (Radke et al., Theor. Appl. Genet. (1988) 75:685; Moloney et al., Plant Cell Reports (1989) 8:238);

soybean (wild), *Glycine canescens* (Rech et al., Plant Cell Reports (1989) 8:33);

strawberry, *Fragaria x ananassa* (Nehra et al., Plant Cell Reports (1990) 9:10;

tomato, *Lycopersicon esculentum* (McCormick et al., Plant Cell Reports (1986) 5:81);

walnut, *Juglans regia* (McGranahan et al., Plant Cell Reports (1990) 8:512);

melon, *Cucumis melo* (Fang et al., 86th Annual Meeting of the American Society for Horticultural Science Hort. Science (1989) 24:89);

grape, *Vitis vinifera* (Colby et al., Symposium on Plant Gene Transfer, UCLA Symposia on Molecular and Cellular Biology J Cell Biochem Suppl (1989) 13D:255;

mango, *Mangifera indica* (Mathews, et al., symposium on Plant Gene Transfer, UCLA Symposia on Molecular and Cellular Biology J Cell Biochem Suppl (1989) 13D:264);

and for the following monocots:

rice, *Oryza sativa* (Shimamoto et al., Nature (1989) 338:274);

rye, *Secale cereale* (de la Pena et al., Nature (1987) 325:274);

maize, (Rhodes et al., Science (1988) 240:204).

In addition regeneration of whole plants from cells (not necessarily transformed) has been observed in

apricot, *Prunus armeniaca* (Pieterse, Plant Cell Tissue and Organ Culture (1989) 19:175);

asparagus, *Asparagus officinalis* (Elmer et al., J. Amer. Soc. Hort. Sci. (1989) 114:1019);

Banana, hybrid *Musa* (Escalant and Teisson, Plant Cell Reports (1989) 7:665);

bean, *Phaseolus vulgaris* (McClean and Grafton, Plant Science (1989) 60:117);

cherry, hybrid Prunus (Ochatt et al., Plant Cell Reports (1988) 7:393);

grape, *Vitis vinifera* (Matsuta and Hirabayashi, Plant Cell Reports, (1989) 7:684;

mango, *Mangifera indica* (DeWald et al., J Amer Soc Hort Sci (1989) 114:712);

melon, *Cucumis melo* (Moreno et al., Plant Sci letters (1985) 34:195);

ochra, *Abelmoschus esculentus* (Roy and Mangat, Plant Science (1989) 60:77; Dirks and van Buggenum, Plant Cell Reports (1989) 7:626);

onion, hybrid *Allium* (Lu et al., Plant Cell Reports (1989) 7:696);

orange, *Citrus sinensis* (Hidaka and Kajikura, Scientia Horiculturae (1988) 34:85);

papaya, *Carrica papaya* (Litz and Conover, Plant Sci Letters (1982) 26:153);

peach, *Prunus persica* and plum, *Prunus domestica* (Mante et al., Plant Cell Tissue and Organ Culture (989) 19:1);

pear, *Pyrus communis* (Chevreau et al., Plant Cell Reports (1988) 7:688; Ochatt and Power, Plant Cell Reports (1989) 7:587);

pineapple, *Ananas comosus* (DeWald et al., Plant Cell Reports (1988) 7:535);

watermelon, *Citrullus vulgaris* (Srivastava et al., Plant Cell Reports (1989) 8:300);

wheat, *Triticum aestivum* (Redway et al., Plant Cell Reports (1990) 8:714).

The regenerated plant are transferred to standard soil conditions and cultivated in a conventional manner.

After the expression cassette is stably incorporated into regenerated transgenic plants, it can be transferred to other plants by sexual crossing.

Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

It may be useful to generate a number of individual transformed plants with any recombinant construct in order to recover plants free from any position effects. It may also be preferable to select plants that contain more than one copy of the introduced recombinant DNA molecule such that high levels of expression of the recombinant molecule are obtained.

As indicated above, it may be desirable to produce plant lines which are homozygous for a particular gene. In some species this is accomplished rather easily by the use of anther culture or isolated microspore culture. This is especially true for the oil seed crop *Brassica napus* (Keller and Armstrong, Z. flanzenzucht 80:100-108, 1978). By using these techniques, it is possible to produce a haploid line that carries the inserted gene and then to double the chromosome number either spontaneously or by the use of colchicine. This gives rise to a plant that is homozygous for the inserted gene, which can be easily assayed for if the inserted gene carries with it a suitable selection marker gene for detection of plants carrying that gene. Alternatively, plants may be self-fertilized, leading to the production of a mixture of seed that consists of, in the simplest case, three types, homozygous (25%), heterozygous (50%) and null (25%) for the inserted gene. Although it is relatively easy to score null plants from those that contain the gene, it is possible in practice to score the homozygous from heterozygous plants by southern blot analysis in which careful attention is paid to the loading of exactly equivalent amounts of DNA from the mixed population, and scoring heterozygotes by the intensity of the signal from a probe specific for the inserted gene. It is advisable to verify the results of the southern blot analysis by allowing each independent transformant to self-fertilize, since additional evidence for homozygosity can be obtained by the simple fact that if the plant was homozygous for the inserted gene, all of the subsequent plants from the selfed seed will contain the gene, while if the plant was heterozygous for the gene, the generation grown from the selfed seed will contain null plants. Therefore, with simple selfing one can easily select homozygous plant lines that can also be confirmed by southern blot analysis.

Creation of homozygous parental lines makes possible the production of hybrid plants and seeds which will contain a modified sweet component. Transgenic homozygous parental lines are maintained with each parent containing either the first or second recombinant DNA sequence operably linked to a promoter. Also incorporated in this scheme are the advantages of

growing a hybrid crop, including the combining of more valuable traits and hybrid vigor.

It has been determined that ideal sweetness in fruits and vegetables can be achieved when sweet proteins comprise about 1% of the total protein normally present, which is generally about 1%. Thus, the present invention is directed towards either supplementing the protein content of fruits and vegetables to achieve this level of sweet protein content or to maintain this level if it is already present. Measurement of the taste of the transgenic foodstuffs of the invention can be accomplished by °Brix as earlier described, by measurement of total protein content or by taste panels.

The above specification sets forth many preferred embodiments which are not intended to limit the scope of the invention in any manner. It has therefore been demonstrated that the present invention accomplishes at least all of its stated objectives. All references, publications and patents recited herein are expressly incorporated by reference in their entirety.

What is claimed is:

1. An expression construct for production of transgenic plants that will produce foodstuffs having a modified sweet protein component comprising: a recombinant DNA sequence which encodes upon expression a brazzein sweet protein; and a regulatory promoter operably linked to said gene said promoter being capable of directing expression of said gene in a plant organ.

2. The expression construct of claim 1 wherein said promoter is an organ specific promoter.

3. The expression construct of claim 1 wherein said promoter is an inducible promoter.

4. The expression construct of claim 1 wherein said promoter is the E8 promoter.

5. The expression construct of claim 1 wherein said promoter is an constitutive promoter.

6. The expression construct of claim 1 wherein said foodstuff is a melon.

7. A nucleic acid vector comprising the expression construct of claim 1.

8. The vector of claim 7 wherein said vector is a cloning vector.

9. The vector of claim 7 wherein said vector is an expression vector.

10. The vector of claim 7 further comprising a marker gene for selection of transformed cells.

11. The vector of claim 12 wherein said marker gene is selected from the group consisting of an ampicillin resistance gene, a tetracycline resistance and a hygromycin resistance gene.

12. The vector of claim 7 further comprising a polyadenylation signal.

13. A prokaryotic or eukaryotic host cell transformed with the nucleic acid vector of claim 7.

14. A transgenic plant comprising a plant cell or ancestor thereof which has been transformed with the vector of claim 7.

15. A method of producing a hybrid seed which, will produce a plant with a modified sweet component of flavor comprising: pollinating a homozygous parent plant which has been transformed or which ancestor thereof has been transformed with a DNA sequence which encodes upon expression brazzein, wherein said DNA sequence is operably linked to a promoter capable of inducing expression in a plant organ, and harvesting said seed.

16. The method of claim 15 wherein said promoter is a fruit specific promoter.

17. A hybrid seed produced by the method of claim 15.

18. A foodstuff with an increased sweet component of flavor produced by plant which contains a heterologous DNA sequence which encodes upon expression brazzein.

19. The foodstuff of claim 18 wherein said foodstuff is a vegetable.

20. The food stuff of claim 18 wherein said foodstuff is a fruit.

21. A method of producing plant foodstuff with an increased sweet component comprising: transforming a plant cell with a DNA sequence which encodes brazzein operably linked to a promoter capable of inducing expression in a plant organ; generating a plant from said transformed cell, and; harvesting the foodstuff produced by said plant.

22. The method of claim 21 wherein said plant is a melon plant.

23. The method of claim 21 wherein said promoter is a fruit specific promoter.

24. The method of claim 21 wherein said promoter is the E8 promoter.

# INTERNATIONAL SEARCH REPORT

Inte nal Application No
PCT/US 97/07877

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C12N15/29 A01H5/00 A23L1/221 A23L1/236

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | WO 95 31547 A (WISCONSIN ALUMNI RES FOUND) 23 November 1995 see page 4, line 17 - line 27 --- | 1-24 |
| Y | WO 92 01790 A (UNIV CALIFORNIA ;LUCKY BIOTECH CORP (US)) 6 February 1992 see the whole document --- | 1-24 |
| A | WO 94 19467 A (WISCONSIN ALUMNI RES FOUND) 1 September 1994 --- | 1-24 |
| A | BIOTECHNOLOGY, vol. 10, no. 5, May 1992, pages 561-564, XP002036974 PENARRUBIA, L., ET AL.: "Production of the sweet protein monellin in transgenic plants." see the whole document --- | 1-24 |

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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| Date of the actual completion of the international search | Date of mailing of the international search report |
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| European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. ( + 31-70) 340-2040, Tx. 31 651 epo nl, Fax ( + 31-70) 340-3016 | Maddox, A |

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